Gram Staining Lab

Background and Introduction

The Gram stain procedure was developed in 1884 by Hans Christian Gram, is a staining procedure that is commonly by microbiologists to differentiate bacteria based on the physiological properties of their cell walls. It is one of the most important procedures in the identification for most types of bacterial isolates. By conducting the procedure, a microbiologist will gain the knowledge knowing the Gram reaction of a microorganism and the morphological characteristic of the organism. This will help the microbiologist rules out a large number of other bacterial species that will ultimately save them unnecessary work and time. With that said, it is time to discuss the three different types of cell walls that bacteria can have. The three types of cells are Gram-positive, Gram-negative and Acid-Fast. This Gram staining procedure will allow you to differentiate between Gram-positive and Gram-negative cell walls, NOT Acid-fast cell walls.

Pre-Lab Questions:

1. Describe the components of a Gram-positive cell wall
2. Describe the components of a Gram-negative cell wall
3. Draw the Gram-positive and Gram-negative cell wall.

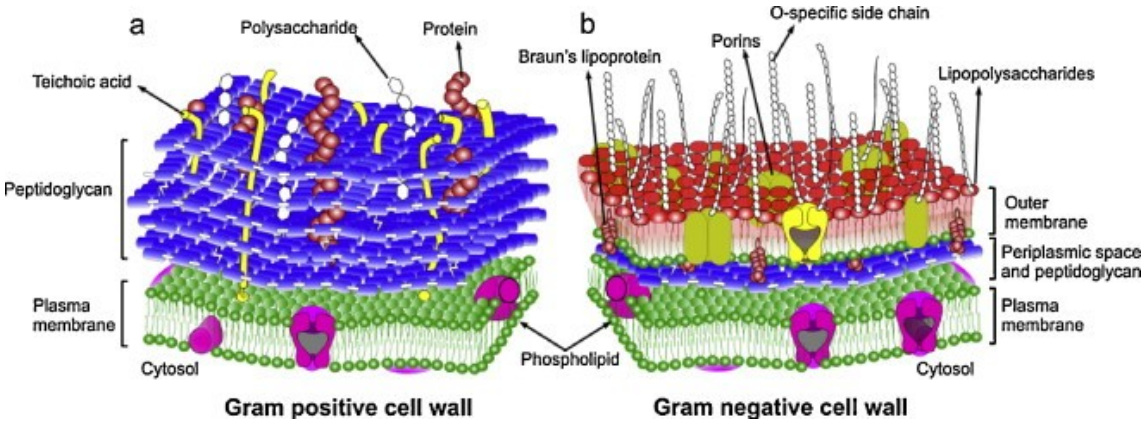
The bacterial specimens are heat-fixed to the glass slide and a crystal violet dye and iodine are applied. The iodine and dye form purple insoluble complexes within the cell. The specimen is then treated with an alcohol solvent which will extract the dye complex through a permeable cell wall. Gram-positive bacterial cell walls are not permeable to this complex and therefore retain the purple dye. Gram-negative bacterial cell walls allow the dye complex to be extracted in this step and therefore appear colorless until they are counterstained with safranin. Gram-negative bacteria appear pink due to the counterstain.

It is possible to receive a "false Gram-negative" if the gram-positive bacteria are old, dead, or damaged and the cell wall is not intact. There is no equivalent "false Gram-positive," but a false Gram-positive can occur if the decolorization step is not performed.

The major difference between Gram-positive and Gram-negative bacteria is cell wall structure. The Gram-positive bacterial cell wall has two major components, peptidoglycan and teichoic acid. There are also additional polysaccharides and proteins that vary according to species. The outer cell wall is thick and chiefly comprised of murein, a peptidoglycan only found in prokaryotes. The inner surface of this cell wall touches the cell membrane, a lipid bilayer. Gram-positive cell walls have low lipid content, no endotoxin, no periplasmic space, and no porin channel.

Gram-negative bacteria have three layers in their cell wall, not counting periplasmic space. Like Gram-positive bacteria, they are surrounded by a cell membrane, but the peptidoglycan layer is thin and does not directly touch the cell membrane. Additionally, the peptidoglycan layer does not contain teichoic acid, although it does contain a murein lipoprotein which binds this layer to the unique outer cell membrane. This outer cell membrane is a phospholipid bilayer that contains lipopolysaccharide (LPS). LPS is the component of the cell wall, which contains Lipid A, or the endotoxin. Also unique in the Gram-negative outer membrane are porin proteins. This allows for the passage of nutrients between the two walls.

Gram staining is still clinically important as the etiologic agents of many bacterial infections are readily seen on Gram-stained smears of pus or fluids. Medical personnel may use this information to guide management of infection before cultures are available. Below is an images as to what the two different cell walls look like:



Pre-Lab Questions:

Define the following vocabulary terms before you come to lab:

Teichoic acid:

Peptidoglycan:

Lipoteichoic acid:

Cytoplasmic membrane:

Lipopolysaccharide (LPS):

Outer membrane:

Peptidoglycan:

Periplasmic Space:

In the gram staining procedure, the primary stain is crystal violet, and all cells take up the purple crystal violet stain. It is a positively charged stain, otherwise known as basic stain. The color of the stain is violet.

After adding the primary stain, Gram's Iodine is to be added in order to flood the bacterial smears. The iodine acts as a mordant, enhancing the ability of the stain to enter and bind to the bacteria. The iodine is negatively charded and is considered a acidic stain. The iodine has the ability to bind with crystal violet and locks it into peptidoglycan of bacteria. This in turn will intensify the purple color if the microbe is Gram-positive.

The decolorizing agent used in the gram staining procedure is 75% ethanol and 25% acetone, which will help melts the Gram-negative outer membrane. This will act as the differentiating step of the procedure. The Gram-positives will remain unaffected due to their thick peptidoglycan and will remain purple. The Gram-negative species with their rich lipid membranes will become decolorized. It also dehydrates proteins, helping the primary stain to remain in Gram-positive cell walls.

The counter stain then used is Safranin. This is another basic stain that will stain the decolorized Gram-negative cells pink. The Gram-negative microbes will remain purple. Therefore, at the end of the Gram staining procedure, the Gram-positive cells are purple and Gram-negative cells are pink.

Overview of Gram Stain:

Primary Stain	Crystal Violet

Mordant	Gram's Iodine
Decolorizer	75% Ethanol / 25% Acetone
Counter Stain	Safranin

Pre-Lab question:

Solvent Name	Gram-Positive Species Color Present	Gram-Negative Color Present

CULTURES NEEDED:

Nutrient broth tubes or slants of the following:

Escherichia coli

Staphylococcus species

Bacillus species

Materials:

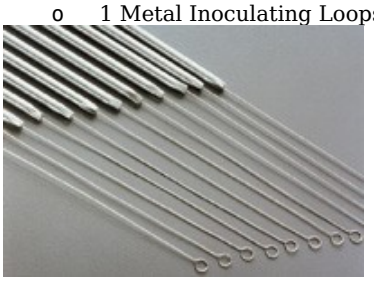
- 1 Wax Pencils

- 1 Distilled Water Bottles

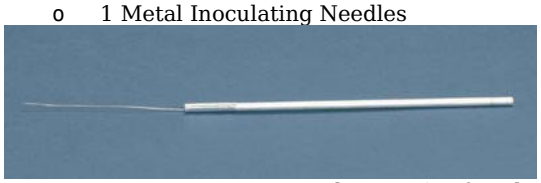
- 1 Disinfectant Bottles

- 1 Test Tube Racks

- 1 Metal Inoculating Loops



- 1 Metal Inoculating Needles



- 1 Bunsen Burners and Hoses (2 of each per table)

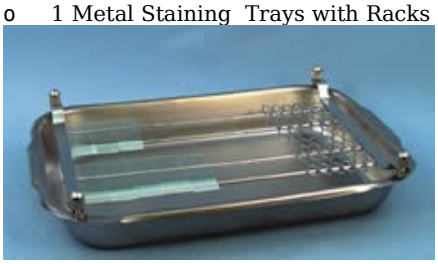
- Boxes of Microscope Slides

- 1 “Waste” 500ml Beakers

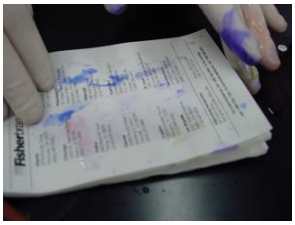
- 1 Gram Staining Kits
  - Each kit should have the following
    - Crystal Violet
    - Gram's Iodine
    - Acetone-Alcohol Decolorizer
    - Safranin

- 1 Optical Lens Paper

- 1 Metal Staining Trays with Racks



- 1 Blotting Paper if available or 1 Large Brown Paper Towels Rolls



PROCEDURE:

Note: This is an individual lab exercise.

1. Prepare a bacterial smear with each organism (i.e. 1-2 loopfuls of each) listed above and heat fix onto the glass slide.

2. Place the slide on a staining tray, and flood the smear with crystal violet. Allow to stain for 60 seconds.

3. Tilt the slide and gently rinse with distilled water until the stain is removed. Flood the smear with Gram's Iodine, and allow to sit for 60 seconds.Tilt the slide and gently rinse with distilled water.

4. IMPORTANT STEP: Tilt the slide and let 2-3 drops of Decolorizer run over the slide. If the last drop is still purple, continue decolorizing, 2-3 drops at a time, until the decolorizer runs clear. Rinse gently with distilled water.

5. Flood the smear with Safranin, and stain for 60 seconds. Tilt the slide and rinse with distilled water.

6. Place the slide in a book of Bibulous paper and blot to dry.

7. Observe the slide under oil immersion, and draw what you see in the results section below.

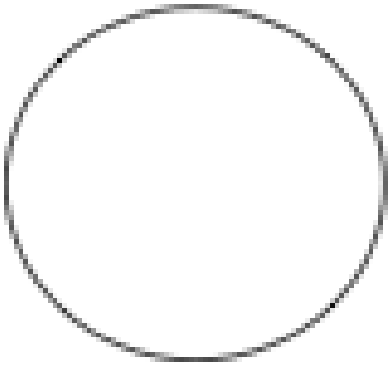
8. Clean your microscope with lens cleaner, paying extra attention to the 40X and 100X objectives. Have your instructor check your microscope to make sure it is clean.

_____ (Instructor's initials)

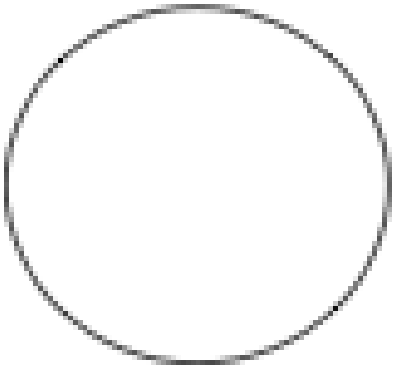
Dispose of the liquid stain waste in the designated waste receptacle in the front and back of the laboratory. Dispose of glass slides in glass waste container. Disinfect your bench and place all materials back to where you found them. In addition, please give your cultures back to your instructor.

Data/Results

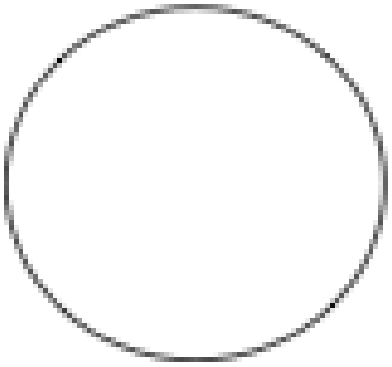
Using colored pencils, draw and label *Staphylococcus species, Bacillus species*, and *E. coli* in the circles below. In addition, write down what you physically see as well. Describe the morphological characteristics of each microbe.



Staphylococcus species at 1000X



Bacillus species at 1000x



E. coli species at 1000x

Post-Lab Questions

1. Describe several advantages of differential staining procedures compared with simple staining techniques.

2. Give the purpose of each of the following reagents in a differential staining procedure:

Primary stain:

Counter stain:

Decolorizing agent:

Mordant:

3. Why is it important for the counter stain to be a lighter color than the primary stain?

4. The substances listed below are used in various differential staining techniques. Which of the following lists the correct order for the solutions used in the Gram stain?
   1. Alcohol
   2. Gram's iodine
   3. Carbol fuchsin
   4. Crystal violet
   5. Methyl red
   6. Methylene blue
   7. Safranin (or basic fuchsin)

   a. 1 - 3 - 4 - 2
   b. 1 - 7 - 2 - 4
   c. 4 - 2 - 1 - 7
   d. 1 - 4 - 3 - 6
   e. 4 - 7 - 1 - 5

5. What color would you expect the *Staphylococcus species* would be if the iodine step was accidently skipped in the Gram-staining procedure? Why?